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Abstract

Background: The medial and maxillary aspects of the upper lip originate at separate embryonic stages and therefore may experience different maternal exposure patterns which may affect methylation. Based on this hypothesis, we investigated the level of methylation of the methylene tetrahydrofolate reductase promoter gene (m*MTHFR*) in tissues from cleft lip, and m*MTHFR* levels by *MTHFR* c.677C>T genotype. We further investigated whether m*MTHFR* mitigates the effect of smoking on long interspersed nuclear element (LINE-1) methylation in these tissues.

Methods. DNA extracted from medial and lateral tissues of 26 infants with non-syndromic cleft lip with or without cleft palate (nsCL/P) was bisulfite converted and m*MTHFR* was measured on a pyrosequenser. LINE-1 methylation and *MTHFR* c.677C>T genotype data were obtained in our previous study.

Results. There was no substantial difference in m*MTHFR* (p=0.733) and LINE-1 (p=0.148) between the two tissues. m*MTHFR* was not influenced by *MTHFR* c.677C>T genotype, but there was suggestive evidence that the difference was larger among infants exposed to maternal smoking compared to non-exposed. LINE-1 methylation differences were significant (p=0.025) in infants born to non-smoking mothers, but this was not apparent (p=0.872) in infants born to mothers who smoked. Our Pearson's correlation analysis suggested a weak inverse association between m*MTHFR* and LINE-1 (r= -0.179; p=0.381).

Conclusion. Our preliminary observation of differences in patterns of m*MTHFR* levels in lip tissue suggests the interplay of gene and environment in establishment of methylation in tissues at both sides of cleft lip. This requires investigation in a larger cohort, integrated with metabolic assessment.

Keywords: Non-syndromic cleft lip with or without cleft palate, DNA methylation, *MTHFR* c.677C>T, LINE-1, *MTHFR* promoter methylation

Introduction

Orofacial clefts (OFCs) are collectively among the most common human congenital anomalies that can occur in isolation or as part of a syndrome (Mossey et al., 2011). Some environmental and multiple genetic risk factors have been identified for non-syndromic form of OFCs (Leisle & Marazita, 2013; Khan et al., 2018a, Mossey et al., 2017; Raut et al., 2019; Johnson & Little, 2008; Little, Cardy, & Munger, 2004) but the causes of these defects remain largely unknown.

OFCs develop in early life, when the embryo is extremely susceptible to perturbation of the in-utero environment (Dixon, Marazita, & Beaty, 2010). Among environmental factors, tobacco smoking has been found to influence facial morphology (Xuan et al., 2016), and is reported to be the most consistent and strongest risk factor for OFCs (Raut et al., 2019). Such perturbation of the early life environment affects developmental programming in the embryo, with sustained changes potentially detectable in tissues from medial nasal and maxillary sides of the upper lip in cases with non-syndromic cleft lip with or without cleft palate (nsCL/P) as observed by our group in recent studies (Khan et al., 2018b, Khan et al., 2018c, Khan et al., 2019a, Khan et al., 2019b).

Methylene tetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme in the one-carbon cycle, a pathway that is critical to metabolism of folate. Folate is a specific nutrient involved in development of craniofacial structures (Jiang, Bush, & Lidral, 2006) and provides methyl group for DNA methylation (Sinclair et al., 2007). MTHFR activity is mainly regulated by the combination of two mechanistic aspects – 1) variants within the gene that essentially acts at the level of enzyme activity and specificity; 2) methylation of the gene promoter that mainly affects level of expression. Both the c.677C>T (rs1801133) and c.1298A>C (rs1801131) variants of the *MTHFR* gene have been demonstrated to reduce enzyme activity (Liew & Gupta, 2015; van der Put et al., 1998). The effect of both these variants have been investigated in relation to nsCL/P in the index child, and/or one or both parents but the results from these studies have been inconclusive (Rai, 2018; Mossey, & Little, 2002; Reutter et al., 2008; Shaw, Todoroff, Finnell, Rozen, & Lammer, 1999; Zhou et al., 2020). Our group; however, considered an alternative mechanism involving DNA methylation to decipher the role of *MTHFR* gene variants in

nsCL/P, and found that a variant in *MTHFR* gene plays a role in the establishment of methylation in cleft lip tissues (Khan et al., 2019b).

We know that methylation within *MTHFR* promoter (m*MTHFR*) contributes to variation in MTHFR protein activity similar to that conferred by *MTHFR* variants (Coppede, Denaro, Tannorella, & Migliore, 2016), and has been shown to contribute to many developmental (Asim, Agarwal, Panigrahi, Sai; yed, & Bakshi, 2017; Coppede et al., 2016) and pregnancy related disorders (Ge et al., 2015; Mishra et al., 2019). However, to our knowledge, there appears to be no evidence of information regarding the methylation profile of m*MTHFR*, or the contribution of *MTHFR* variants to *MTHFR* methylation level in nsCL/P. Therefore, we undertook this preliminary study to assess the level of m*MTHFR*, and further investigate the relationship between *MTHFR* c.677C>T variant and m*MTHFR* utilizing tissues from medial and lateral aspects of the upper lip in individuals with nsCL/P.

m*MTHFR* could also be involved in differences in regulation of methylation repair activity and hence might contribute to individual differences by altering enzyme activity. This could either affect the availability of activated methyl group or increase the rate of loss of methylation (over time) in response to exposures associated with demethylation such as cigarette smoking (Beach et al., 2017). Alternatively, when MTHFR is more active, the availability of methyl group is more likely enhanced, potentially alleviating the impact of exposures such as smoking that would otherwise cause demethylation (Beach et al., 2017; Stover, 2009). Intrigued by this concept, we examined whether m*MTHFR* in the indexed infant could mitigate the adverse effects of active maternal smoking exposures, and potentially be reflected as changes in LINE-1 methylation level – widely accepted to be a proxy for overall genomic DNA methylation content (Lisanti et al., 2013).

Materials and Methods

Tissue samples from the medial and lateral sides of cleft lip were collected from 26 cases with nsCL/P that were recruited between 2016 and 2018 in the Centre for Orofacial Clefts and Craniofacial Anomalies, San Paolo Hospital, Milan, Italy (PENTACLEFT: prot. no. 08–2011). Our sample included 13 female and 13 male cases. Fifteen cases had cleft lip

and 10 had cleft lip and palate (phenotypic data missing for one case – due to mismatching). Among the mothers of these 26 cases, fifteen were non-smokers and eight actively smoked during the periconceptional period – 3 months before to 3 months after conception (smoking data missing for three mothers – due to non-response on survey).

The DNA extracted from tissues were bisulfite converted and methylation of the *MTHFR* gene promoter was measured using PyroMark Q96 predesigned CpG assay (#PM00000091) on a PyroMark Q96 ID pyrosequenser (Qiagen GmbH, Hilden, Germany), with minor modification of the method previously described (Khan et al., 2018b). Briefly, the amplification of bisulfite converted DNA was performed by PCR with *MTHFR* primer, and pyrosequencing done using *MTHFR* specific sequencing primers. The predesigned assay contained the following sequence 5'-GGTCACTGAGTCAC**C**GATGGGGG**C**GAGGAYA**C**GGGC-3' (prior to bisulfite conversion) including 3 CpG sites to assess in promoter region of *MTHFR*. The nucleotide dispensation order was: TGTCATGATGATATCGAGTGGTCGAGATATCG. LINE-1 methylation and *MTHFR* c.677C>T genotype data for this cohort were obtained in our previous study (Khan et al., 2019b). The Kolmogorov-Smirnov test showed that the data were normally distributed, hence, parametric comparison of within and between samples were performed using Student's *t*-test. In addition, we report parametric effect size estimate (Hedge's g_s) associated with independent sample Student's *t*-test, and Pearson's correlation to determine relationship between m*MTHFR* and LINE-1 (Pautz, Olivier, & Steyn, 2018; McLeod, 2019). Considering the total of 26 cases included in this study, we calculated statistical power using G*POWER software 3.1.9.2 version (Faul, Erdfelder, Lang, & Buchner, 2007). Considering a two-tailed *t*-test and an equally serious α & β error (β/α = 1), an effect size dz of 0.2, we calculated the power (1-β-error) of 0.60.

Results

We found a non-significant difference of 1.3% in m*MTHFR* between medial and lateral tissues (p=0.733; Table 1). m*MTHFR* was not influenced by *MTHFR* c.677C>T genotype (Table 2). Similarly, LINE-1 methylation was not significantly different (1.7%) across tissues in this cohort (p=0.148; Table 1).

To examine the role of m*MTHFR* as a source in compensating for the effect of smoking on LINE-1 methylation level, we compared the level of methylation in *MTHFR* and LINE-1 in medial and lateral tissues between infants born to mothers who smoked in the periconceptional period and infants of non-smoking mothers. Among infants exposed to smoking, the difference in m*MTHFR* was larger (6.1%) but showed a lowered level of methylation (p=0.293; Table 1) compared to infants born to non-smoking mothers in whom m*MTHFR* was similar (~38%) in the two tissues (p=0.866; Table 1). Interestingly, LINE-1 methylation differences were significant (p=0.025) in infants born to non-smoking mothers; this however, was not apparent (p=0.872) in infants born to mothers who smoked, with medial and lateral tissues showing equal level (72%) of methylation (Table 1).

Comparisons between these groups were non-significant for both m*MTHFR* (p=0.554) and LINE-1 (p=0.209). We also calculated the effect size (ES) for between comparisons and found a small ES for m*MTHFR* (gs=0.26). While LINE-1 showed medium (gs=0.56) ES, our Pearson's correlation analysis suggested a weak inverse association between m*MTHFR* and LINE-1 (r= -0.179; p=0.381).

Discussion

In this preliminary study, we found a small non-significant difference in m*MTHFR* and LINE-1 methylation across medial and lateral tissues. A difference in m*MTHFR* was observed in infants of mothers who smoked but not among infants born to non-smoking mothers. By contrast, a significant difference in LINE-1 methylation was apparent in infants born to non-smoking mothers, but not in infants born to smoking mothers.

Our observation of small changes in methylation in m*MTHFR* and LINE-1 is compatible with reports that the magnitude of epigenetic effect associated with exposure in children is generally small; large changes may not be compatible with continued development (Breton et al., 2017). Hence, a small imbalance in methylation in progeny cells of the medial and maxillary tissues might result in an apparently small distinction between sufficient and insufficient methylation. Insufficient methylation might in turn interfere with the fusion process, so leading to the development of a cleft lip. Persistence of this small imbalance throughout pregnancy and into the postnatal period would be manifested

as differences in tissues from medial and lateral side of cleft lip. Such a difference could therefore provide insight into epigenetic effects of early life environmental exposures (Richmond et al., 2017).

The pattern of m*MTHF*R levels suggests that nearly equal levels of folate are available for tissues developing at distinct embryonic periods, but the level of availability could likely be influenced by external factors such as smoking (Nafee, Farrell, Carroll, Fryer, & Ismail, 2008). Importantly, we found lower m*MTHFR* in the smoking group that suggests an increase in folate availability. This increased folate availability might provide methyl-group to mitigate/overcome the effect of smoking resulting in the observation of nearly equal levels of LINE-1 methylation in the medial and lateral tissues (72%). The lower m*MTHFR* level in the smoking group further suggests smoking-associated demethylation at a single gene promoter, whereas there was little difference in global methylation. This is in line with the suggestion that small changes in global methylation of developing tissues might have substantial effects in the longer term (Breton et al., 2017). Pearson's correlation analysis showed a weak association between m*MTHFR* and LINE-1. This provides some support for a role of m*MTHFR* in moderating epigenetic response to smoking, and our previous findings that lip tissues are highly responsive to maternal environmental exposures (Khan et al., 2018b). We acknowledge that our results are based on small sample size, because collecting tissues from the cleft cases presents considerable challenges (Stock et al., 2016). We did not correct for multiple comparisons because reducing the risk of type I error can be at the expense of increasing type II error, and because of the preliminary nature of the study, identifying hypotheses for further investigation (Perneger 1998; Armstrong, 2014). A limitation of using tissue from the clefts is the difficulty of obtaining an appropriate reference group from which lip tissue samples could be collected. This problem arises from concerns about ethical issues and selection bias and is highly likely to be encountered in other studies.

Our result is consistent with previous reports involving a large number of healthy Italian participants showing no association of *MTHFR* c.677C>T with m*MTHFR* indicating that c.677C>T variant does not act as a *cis* regulatory element to regulate its own gene promoter (Piras et al., 2020; Coppede et al., 2019; Ni et al., 2017), although there are reports that *MTHFR* c.677C>T genotype influences m*MTHFR* (Mandaviya et al. 2017;

Nash et al., 2019). Accordingly, we found that m*MTHFR* in TT homozygotes are hypomethylated in both medial and lateral tissues, which seems to reflect a compensatory higher expression of *MTHFR* gene. This is in line with a previous report of the complexity of the effect of *MTHFR* variants on DNA methylation (De Gobbo, Price, Hanna, & Robinson, 2018). A comprehensive metabolic assessment is necessary to advance our understanding of one-carbon nutrients on DNA methylation involved in nsCL/P.

The investigation of LINE-1 methylation in response to smoking does not necessarily reflect changes in methylation at specific loci that have been reported to be influenced by smoking (Andersen, Dogan, Beach, & Philibert, 2015). Hence, for understanding aspects of the apparent mitigating effect of m*MTHFR* on smoking, we in future plan investigation based on larger samples, and genetic loci/CpGs previously identified as being associated with smoking and also implicated in non-syndromic OFCs (Joubert et al., 2016). Another potential limitation of this study is non availability of RNA from these tissues to access relationship between m*MTHFR* and its expression (mRNA level) - which can be modulated by other epigenetic processes such as histone modification and micro-RNAs. In this regard, there is evidence suggesting that miRNAs (miR-324-3p and miR-223), are able to regulate *MTHFR* gene in salivary cells taken from nsCL/P cases (Grassia et al., 2018). Once tissue collections still in process, are completed, we also plan to investigate miRNAs and functional analysis in these tissues to better understand the complex aetiology of nsCL/P.

In conclusion, our study highlights the interplay of gene and environment in moderating the establishment of methylation in medial and maxillary sides of the upper lip tissues. The study requires replication in a larger study, including genes associated with smoking and oral clefts. We consider that the study further champions the potential value of investigating lip tissues, integrated with metabolomics for nutrient assessment, in order to develop a clearer understanding of the aetio-pathogenesis of non-syndromic orofacial clefts.

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Table 1. Mean methylation level (%) at LINE-1 and *MTHFR* gene promoter (m*MTHFR*) in medial and lateral cleft tissues.

Mean ± standard deviation (SD) values of total non-syndromic CL/P cases or cases categorized by smoking and non-smoking, along with mean difference, 95% confidence interval (C.I) and nominal *p*-value of *t*-test.

Infant DNA	**Medial side** Mean ± SD	**Lateral side** Mean ± SD	Mean difference (95% C.I.)	*p*-value
LINE 1 (26)				
Total (n=26)	**73.19** ± 2.57	**71.50** ± 4.65	1.69 (-0.64 to 4.02)	*p*=0.148
Non-smoking (n=15)	**73.78** ± 2.48	**70.22** ± 4.95	3.55 (0.51 to 6.59) ***p*=0.025**	*p*=0.209
Smoking (n=8)	**72.00** ± 2.56	**72.38** ± 5.15	-0.38 (-5.70 to 4.94) *p*=0.872	
m*MTHFR* (26)				
Total (n=26)	**37.04** ± 1.63	**35.69** ± 1.85	1.34 (-6.69 to 9.38)	*p*=0.733
Non-smoking (n=15)	**37.50** ± 1.82	**38.28** ± 1.81	-0.77 (-10.33 to 8.77) *p*=0.866	*p*=0.554
Smoking (n=8)	**31.13** ± 11.50	**25.00** ± 11.73	6.12 (-6.60 to 18.85) *p*=0.293	

Abbreviations: n, number of cases; SD, standard deviation; CI, confidence interval.
Footnote: Maternal smoking data was available for only 23 cases.

Table 2. m*MTHFR* level (%) in medial and lateral cleft lip tissues of total non-syndromic CL/P cases, stratified by *MTHFR* c.677C>T genotype.

MTHFR c.677C>T	**Medial side** Mean ± SD	**Lateral side** Mean ± SD	Mean difference (95% C.I.)	*p*-value*
m*MTHFR* (26)				
CC (n=7)	**44.57** ± 1.46	**37.71** ± 2.11	6.86 (-17.57 to 31.28)	ref.
CT (n=13)	**38.54** ± 16.62	**37.77** ± 18.90	0.7 (-9.97 to 11.51)	*p*=0.649
TT (n=6)	**25.00** ± 12.36	**28.83** ± 15.94	-3.83 (-22.28 to 14.61)	*p*=0.390

Abbreviations: n, number of cases; SD, standard deviation; CI, confidence interval; ref., reference.
*Nominal p-value of comparisons of mean difference between medial and lateral sides considering CC genotype as reference.

MTHFR promoter methylation might mitigate the effect of smoking at the level of LINE-1 in cleft lip tissues – a preliminary study

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Abstract

Background: The medial and maxillary aspects of the upper lip originate at separate embryonic stages and therefore may experience different maternal exposure patterns which may affect methylation. Based on this hypothesis, we investigated the level of methylation of the methylene tetrahydrofolate reductase promoter gene (m*MTHFR*) in tissues from cleft lip, and m*MTHFR* levels by *MTHFR* c.677C>T genotype. We further investigated whether m*MTHFR* mitigates the effect of smoking on long interspersed nuclear element (LINE-1) methylation in these tissues.

Methods. DNA extracted from medial and lateral tissues of 26 infants with non-syndromic cleft lip with or without cleft palate (nsCL/P) was bisulfite converted and m*MTHFR* was measured on a pyrosequenser. LINE-1 methylation and *MTHFR* c.677C>T genotype data were obtained in our previous study.

Results. There was no substantial difference in m*MTHFR* (p=0.733) and LINE-1 (p=0.148) between the two tissues. m*MTHFR* was not influenced by *MTHFR* c.677C>T genotype, but there was suggestive evidence that the difference was larger among infants exposed to maternal smoking compared to non-exposed. LINE-1 methylation differences were significant (p=0.025) in infants born to non-smoking mothers, but this was not apparent (p=0.872) in infants born to mothers who smoked. Our Pearson's correlation analysis suggested a weak inverse association between m*MTHFR* and LINE-1 (r= -0.179; p=0.381).

Conclusion. Our preliminary observation of differences in patterns of m*MTHFR* levels in lip tissue suggests the interplay of gene and environment in establishment of methylation in tissues at both sides of cleft lip. This requires investigation in a larger cohort, integrated with metabolic assessment.

Keywords: Non-syndromic cleft lip with or without cleft palate, DNA methylation, *MTHFR* c.677C>T, LINE-1, *MTHFR* promoter methylation

Introduction

Orofacial clefts (OFCs) are collectively among the most common human congenital anomalies that can occur in isolation or as part of a syndrome (Mossey et al., 2011). Some environmental and multiple genetic risk factors have been identified for non-syndromic form of OFCs (Leisle & Marazita, 2013; Khan et al., 2018a, Mossey et al., 2017; Raut et al., 2019; Johnson & Little, 2008; Little, Cardy, & Munger, 2004) but the causes of these defects remain largely unknown.

OFCs develop in early life, when the embryo is extremely susceptible to perturbation of the in-utero environment (Dixon, Marazita, & Beaty, 2010). Among environmental factors, tobacco smoking has been found to influence facial morphology (Xuan et al., 2016), and is reported to be the most consistent and strongest risk factor for OFCs (Raut et al., 2019). Such perturbation of the early life environment affects developmental programming in the embryo, with sustained changes potentially detectable in tissues from medial nasal and maxillary sides of the upper lip in cases with non-syndromic cleft lip with or without cleft palate (nsCL/P) as observed by our group in recent studies (Khan et al., 2018b, Khan et al., 2018c, Khan et al., 2019a, Khan et al., 2019b).

Methylene tetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme in the one-carbon cycle, a pathway that is critical to metabolism of folate. Folate is a specific nutrient involved in development of craniofacial structures (Jiang, Bush, & Lidral, 2006) and provides methyl group for DNA methylation (Sinclair et al., 2007). MTHFR activity is mainly regulated by the combination of two mechanistic aspects – 1) variants within the gene that essentially acts at the level of enzyme activity and specificity; 2) methylation of the gene promoter that mainly affects level of expression. Both the c.677C>T (rs1801133) and c.1298A>C (rs1801131) variants of the *MTHFR* gene have been demonstrated to reduce enzyme activity (Liew & Gupta, 2015; van der Put et al., 1998). The effect of both these variants have been investigated in relation to nsCL/P in the index child, and/or one or both parents but the results from these studies have been inconclusive (Rai, 2018; Mossey, & Little, 2002; Reutter et al., 2008; Shaw, Todoroff, Finnell, Rozen, & Lammer, 1999; Zhou et al., 2020). Our group; however, considered an alternative mechanism involving DNA methylation to decipher the role of *MTHFR* gene variants in

nsCL/P, and found that a variant in *MTHFR* gene plays a role in the establishment of methylation in cleft lip tissues (Khan et al., 2019b).

We know that methylation within *MTHFR* promoter (m*MTHFR*) contributes to variation in MTHFR protein activity similar to that conferred by *MTHFR* variants (Coppede, Denaro, Tannorella, & Migliore, 2016), and has been shown to contribute to many developmental (Asim, Agarwal, Panigrahi, Sai; yed, & Bakshi, 2017; Coppede et al., 2016) and pregnancy related disorders (Ge et al., 2015; Mishra et al., 2019). However, to our knowledge, there appears to be no evidence of information regarding the methylation profile of m*MTHFR*, or the contribution of *MTHFR* variants to *MTHFR* methylation level in nsCL/P. Therefore, we undertook this preliminary study to assess the level of m*MTHFR*, and further investigate the relationship between *MTHFR* c.677C>T variant and m*MTHFR* utilizing tissues from medial and lateral aspects of the upper lip in individuals with nsCL/P.

m*MTHFR* could also be involved in differences in regulation of methylation repair activity and hence might contribute to individual differences by altering enzyme activity. This could either affect the availability of activated methyl group or increase the rate of loss of methylation (over time) in response to exposures associated with demethylation such as cigarette smoking (Beach et al., 2017). Alternatively, when MTHFR is more active, the availability of methyl group is more likely enhanced, potentially alleviating the impact of exposures such as smoking that would otherwise cause demethylation (Beach et al., 2017; Stover, 2009). Intrigued by this concept, we examined whether m*MTHFR* in the indexed infant could mitigate the adverse effects of active maternal smoking exposures, and potentially be reflected as changes in LINE-1 methylation level – widely accepted to be a proxy for overall genomic DNA methylation content (Lisanti et al., 2013).

Materials and Methods

Tissue samples from the medial and lateral sides of cleft lip were collected from 26 cases with nsCL/P that were recruited between 2016 and 2018 in the Centre for Orofacial Clefts and Craniofacial Anomalies, San Paolo Hospital, Milan, Italy (PENTACLEFT: prot. no. 08–2011). Our sample included 13 female and 13 male cases. Fifteen cases had cleft lip

and 10 had cleft lip and palate (phenotypic data missing for one case – due to mismatching). Among the mothers of these 26 cases, fifteen were non-smokers and eight actively smoked during the periconceptional period – 3 months before to 3 months after conception (smoking data missing for three mothers – due to non-response on survey).

The DNA extracted from tissues were bisulfite converted and methylation of the *MTHFR* gene promoter was measured using PyroMark Q96 predesigned CpG assay (#PM00000091) on a PyroMark Q96 ID pyrosequenser (Qiagen GmbH, Hilden, Germany), with minor modification of the method previously described (Khan et al., 2018b). Briefly, the amplification of bisulfite converted DNA was performed by PCR with *MTHFR* primer, and pyrosequencing done using *MTHFR* specific sequencing primers. The predesigned assay contained the following sequence 5'-GGTCACTGAGTCAC**C**GATGGGGG**C**GAGGAYA**C**GGGC-3' (prior to bisulfite conversion) including 3 CpG sites to assess in promoter region of *MTHFR*. The nucleotide dispensation order was: TGTCATGATGATATCGAGTGGTCGAGATATCG. LINE-1 methylation and *MTHFR* c.677C>T genotype data for this cohort were obtained in our previous study (Khan et al., 2019b). The Kolmogorov-Smirnov test showed that the data were normally distributed, hence, parametric comparison of within and between samples were performed using Student's *t*-test. In addition, we report parametric effect size estimate (Hedge's g_s) associated with independent sample Student's *t*-test, and Pearson's correlation to determine relationship between m*MTHFR* and LINE-1 (Pautz, Olivier, & Steyn, 2018; McLeod, 2019). Considering the total of 26 cases included in this study, we calculated statistical power using G*POWER software 3.1.9.2 version (Faul, Erdfelder, Lang, & Buchner, 2007). Considering a two-tailed *t*-test and an equally serious α & β error (β/α = 1), an effect size dz of 0.2, we calculated the power (1-β-error) of 0.60.

Results

We found a non-significant difference of 1.3% in m*MTHFR* between medial and lateral tissues (p=0.733; Table 1). m*MTHFR* was not influenced by *MTHFR* c.677C>T genotype (Table 2). Similarly, LINE-1 methylation was not significantly different (1.7%) across tissues in this cohort (p=0.148; Table 1).

To examine the role of m*MTHFR* as a source in compensating for the effect of smoking on LINE-1 methylation level, we compared the level of methylation in *MTHFR* and LINE-1 in medial and lateral tissues between infants born to mothers who smoked in the periconceptional period and infants of non-smoking mothers. Among infants exposed to smoking, the difference in m*MTHFR* was larger (6.1%) but showed a lowered level of methylation (p=0.293; Table 1) compared to infants born to non-smoking mothers in whom m*MTHFR* was similar (~38%) in the two tissues (p=0.866; Table 1). Interestingly, LINE-1 methylation differences were significant (p=0.025) in infants born to non-smoking mothers; this however, was not apparent (p=0.872) in infants born to mothers who smoked, with medial and lateral tissues showing equal level (72%) of methylation (Table 1).

Comparisons between these groups were non-significant for both m*MTHFR* (p=0.554) and LINE-1 (p=0.209). We also calculated the effect size (ES) for between comparisons and found a small ES for m*MTHFR* (gs=0.26). While LINE-1 showed medium (gs=0.56) ES, our Pearson's correlation analysis suggested a weak inverse association between m*MTHFR* and LINE-1 (r= -0.179; p=0.381).

Discussion

In this preliminary study, we found a small non-significant difference in m*MTHFR* and LINE-1 methylation across medial and lateral tissues. A difference in m*MTHFR* was observed in infants of mothers who smoked but not among infants born to non-smoking mothers. By contrast, a significant difference in LINE-1 methylation was apparent in infants born to non-smoking mothers, but not in infants born to smoking mothers.

Our observation of small changes in methylation in m*MTHFR* and LINE-1 is compatible with reports that the magnitude of epigenetic effect associated with exposure in children is generally small; large changes may not be compatible with continued development (Breton et al., 2017). Hence, a small imbalance in methylation in progeny cells of the medial and maxillary tissues might result in an apparently small distinction between sufficient and insufficient methylation. Insufficient methylation might in turn interfere with the fusion process, so leading to the development of a cleft lip. Persistence of this small imbalance throughout pregnancy and into the postnatal period would be manifested

as differences in tissues from medial and lateral side of cleft lip. Such a difference could therefore provide insight into epigenetic effects of early life environmental exposures (Richmond et al., 2017).

The pattern of m*MTHF*R levels suggests that nearly equal levels of folate are available for tissues developing at distinct embryonic periods, but the level of availability could likely be influenced by external factors such as smoking (Nafee, Farrell, Carroll, Fryer, & Ismail, 2008). Importantly, we found lower m*MTHFR* in the smoking group that suggests an increase in folate availability. This increased folate availability might provide methyl-group to mitigate/overcome the effect of smoking resulting in the observation of nearly equal levels of LINE-1 methylation in the medial and lateral tissues (72%). The lower m*MTHFR* level in the smoking group further suggests smoking-associated demethylation at a single gene promoter, whereas there was little difference in global methylation. This is in line with the suggestion that small changes in global methylation of developing tissues might have substantial effects in the longer term (Breton et al., 2017). Pearson's correlation analysis showed a weak association between m*MTHFR* and LINE-1. This provides some support for a role of m*MTHFR* in moderating epigenetic response to smoking, and our previous findings that lip tissues are highly responsive to maternal environmental exposures (Khan et al., 2018b). We acknowledge that our results are based on small sample size, because collecting tissues from the cleft cases presents considerable challenges (Stock et al., 2016). We did not correct for multiple comparisons because reducing the risk of type I error can be at the expense of increasing type II error, and because of the preliminary nature of the study, identifying hypotheses for further investigation (Perneger 1998; Armstrong, 2014). A limitation of using tissue from the clefts is the difficulty of obtaining an appropriate reference group from which lip tissue samples could be collected. This problem arises from concerns about ethical issues and selection bias and is highly likely to be encountered in other studies.

Our result is consistent with previous reports involving a large number of healthy Italian participants showing no association of *MTHFR* c.677C>T with m*MTHFR* indicating that c.677C>T variant does not act as a *cis* regulatory element to regulate its own gene promoter (Piras et al., 2020; Coppede et al., 2019; Ni et al., 2017), although there are reports that *MTHFR* c.677C>T genotype influences m*MTHFR* (Mandaviya et al. 2017;

Nash et al., 2019). Accordingly, we found that m*MTHFR* in TT homozygotes are hypomethylated in both medial and lateral tissues, which seems to reflect a compensatory higher expression of *MTHFR* gene. This is in line with a previous report of the complexity of the effect of *MTHFR* variants on DNA methylation (De Gobbo, Price, Hanna, & Robinson, 2018). A comprehensive metabolic assessment is necessary to advance our understanding of one-carbon nutrients on DNA methylation involved in nsCL/P.

The investigation of LINE-1 methylation in response to smoking does not necessarily reflect changes in methylation at specific loci that have been reported to be influenced by smoking (Andersen, Dogan, Beach, & Philibert, 2015). Hence, for understanding aspects of the apparent mitigating effect of m*MTHFR* on smoking, we in future plan investigation based on larger samples, and genetic loci/CpGs previously identified as being associated with smoking and also implicated in non-syndromic OFCs (Joubert et al., 2016). Another potential limitation of this study is non availability of RNA from these tissues to access relationship between m*MTHFR* and its expression (mRNA level) - which can be modulated by other epigenetic processes such as histone modification and micro-RNAs. In this regard, there is evidence suggesting that miRNAs (miR-324-3p and miR-223), are able to regulate *MTHFR* gene in salivary cells taken from nsCL/P cases (Grassia et al., 2018). Once tissue collections still in process, are completed, we also plan to investigate miRNAs and functional analysis in these tissues to better understand the complex aetiology of nsCL/P.

In conclusion, our study highlights the interplay of gene and environment in moderating the establishment of methylation in medial and maxillary sides of the upper lip tissues. The study requires replication in a larger study, including genes associated with smoking and oral clefts. We consider that the study further champions the potential value of investigating lip tissues, integrated with metabolomics for nutrient assessment, in order to develop a clearer understanding of the aetio-pathogenesis of non-syndromic orofacial clefts.

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